Dynamic analysis of physiological properties of *Torulaspora delbrueckii* in wine fermentations and its incidence on wine quality

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Abstract This work examines the physiology of a new commercial strain of *Torulaspora delbrueckii* in the production of red wine following different combined fermentation strategies. For a detailed comparison, several yeast metabolites and the strains implantation were measured over the entire fermentation period. In all fermentations in which *T. delbrueckii* was involved, the ethanol concentration was reduced; some malic acid was consumed; more pyruvic acid was released, and fewer amounts of higher alcohols were produced. The sensorial properties of final wines varied widely, emphasising the structure of wine in sequential fermentations with *T. delbrueckii*. These wines presented the maximum overall impression and were preferred by tasters. Semi-industrial assays were carried out confirming these differences at a higher scale. No important differences were observed in volatile aroma composition between fermentations. However, differences in mouthfeel properties were observed in semi-industrial fermentations, which were correlated with an increase in the mannoprotein content of red wines fermented sequentially with *T. delbrueckii*.

Keywords *Torulaspora delbrueckii* · Manno proteins · Glyceropyruvic pathway · Malic acid · Pyruvic acid · Combined fermentation

Introduction

Many research groups are currently studying non-*Saccharomyces* yeasts (Comitini et al. 2011; Contreras et al. 2014; Garde-Cerdán and Ancín-Azpilicueta 2006; Jolly et al. 2006) due to their unique physiological metabolic properties, which may be advantageous in winemaking. The presence of non-*Saccharomyces* wild yeasts in fermentations has been associated, traditionally, with high levels of acetic acid and other off-flavours. Nevertheless, nowadays, researchers and winemakers are aware of the influence of non-*Saccharomyces* in wine aroma complexity (Egli et al. 1998; Esteve-Zarzoso et al. 1998; Fleet 2003, 2008; Fleet and Heard 1993; Gil et al. 1996; Henick-Kling et al. 1998; Lambrechts and Pretorius 2000; Romano et al. 2003; Viana et al. 2008). The difficulty with which non-*Saccharomyces* wine yeast finishes the alcoholic fermentation requires the development of combined fermentation with *Saccharomyces cerevisiae* as a binding partner. Some enzymatic activities related to aroma enhancement (glycosidases and β-lyase for terpene and thiol release, respectively) and the release of some interesting products such as glycerol and mannoproteins, among others, are the highlights that justify the interest in these mixed fermentations (Ciani et al. 2010; Rojas et al. 2001). In this context, combined fermentations are a very useful tool to improve wine fermentations in which aromatic complexity of spontaneous fermentations and the safety of industrial targeted fermentations are joined (Ciani et al. 2010; Romano et al. 2003).

Some studies have analysed the use and influence of different non-*Saccharomyces* species in wine fermentations, such as *Kloeckera apiculata* (Herraiz et al. 1990), other

apiculated yeasts like *Hanseniaspora uvarum* (Zironi et al. 1993), *Torulaspora delbrueckii*, *Kluyveromyces thermotolerans*, *Hansenula anomala*, and *Metschnikowia pulcherrima* (Ciani et al. 2006; Izquierdo-Cañas et al. 2011, 2014; Oro et al. 2014).

Despite that studies of industrial or semi-industrial use of *T. delbrueckii* and its repercussion on wine quality are scarce, most scientific studies report its relationship with wines with low acetic acid content and great mouthfeel properties (Bely et al. 2008). Furthermore, the fermentative capacity of *T. delbrueckii* (Quirós et al. 2014) allows its implantation at the beginning of fermentation process, contrary to other strictly oxidising non-*Saccharomyces* yeasts. At the same time that several authors are studying the potential use of non-*Saccharomyces* yeasts in wine fermentations (De Benedictis et al. 2010; Domizio et al. 2011; Viana et al. 2008), the enology industry has been able to accept this trend, and most wine yeast distribution companies already have non-*Saccharomyces* strains for its use in winery.

The possibility to modulate the flavour and style of wine by different fermentation strategies forced the study on all possible combinations of non-*Saccharomyces* and *Saccharomyces* yeast strains (Azzolini et al. 2012). In this sense, most of studies analyse fermentations carried out with non-*Saccharomyces* strains by itself, with mixed fermentations by simultaneous and sequential inoculation, comparing all of them with the alcoholic fermentation with *S. cerevisiae* by itself.

This study aims to validate the industrial use of a new commercial strain of *T. delbrueckii* from Agrovin S.A., studying their physiology throughout fermentation in order to explain the chemical composition, aromatic profile and sensorial properties of the red Tempranillo wines produced by different mixed cultures of the strain *T. delbrueckii* NSA-1 with *S. cerevisiae*.

Most of studies reported to analysing the properties and that advantages of some non-*Saccharomyces* yeast are developed following a microvinification trend, but results are rarely validated in an industrial or semi-industrial scale, questioning its potential applicability (Jolly et al. 2014) due to the influence of scale on yeast gene expression (Rossouw et al. 2012). In order to validate microvinification results in this study, semi-industrial fermentation was carried out in 100-L stainless tanks.

Materials and methods

Microorganisms

Yeast strains and molecular identification

S. cerevisiae CT007 and *T. delbrueckii* NSA-1 Viniferm NS-TD were obtained from the Agrovin S.A. (Alcázar de San Juan, Spain) collection and identified by using molecular methods as follows. Yeast isolates were identified by sequence analysis of the 26S large subunit rRNA gene. Total genomic DNA was extracted using the isopropanol method (Querol et al. 1992), and DNA for sequencing was amplified using an Eppendorf Mastercycler apparatus as described by Kurtzman and Robnett (1997) with forward NL-1 primer (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse NL-4 primer (5′-GGT CCG TGT TTC AAG ACG G′). Sequences obtained to identify yeasts were analysed and compared by BLAST-search (GenBank;www.ncbi.nlm.nih.gov). The 26S rRNA nucleotide sequences has been submitted to Genbank-NCBI under accession numbers KM434246 (*S. cerevisiae* CT007) and KM434245 (*T. delbrueckii* NSA-1).

Additionally, *S. cerevisiae* CT007 identification was confirmed by the polymerase chain reaction amplification of the interdelta region of *S. cerevisiae* (Legras and Karst 2003) using delta12 (5′-TCAACAATGGAATCCCAAC-3′) and delta21 (5′- CATCTTAACACCGTATATGA-3′) primers.

Characterisation of yeast strains

β-Glucosidase activity was evaluated as reported by Rosi et al. (1994), on a medium containing 0.5 % cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose), 0.67 % yeast nitrogen base (Difco) and 2 % agar. Yeast strains were inoculated as above and incubated at 28 °C for 3 days. A significant growth of the colonies indicated the presence of β-glucosidase activity. Additionally, β-D-xylosidase and α-L-arabinofuranosidase activities were evaluated using the correspondent methylumbelliferyl-conjugated substrates (methylumbelliferyl-β-D-xylopyranoside and methylumbelliferyl-α-L-arabinofuranosidase, respectively), according to the method described by Manzanares et al. (1999). Strains of *T. delbrueckii* CECT 10676 from the Spanish Type Culture Collection (CECT Valencia, Spain) and *Rhodotorula mucilaginosa* NSG-61 from the Complutense Yeast Collection (CYC Madrid, Spain) were used as were used as negative and positive controls, respectively.

Production of hydrogen sulfide was evaluated by using a modification of the lead acetate method (Linderholm et al. 2008). This method detects volatile H_2S in the headspace of the fermentation in a culture medium containing 1.17 % yeast carbon base (Difco), 4 % glucose anhydrous, and 0.5 % ammonium sulfate. Yeasts were grown at 28 °C for 3 days in 96-well microplates containing 200 μl of medium with orbital agitation (200 rpm). Hydrogen sulfide formation was initially detected by using paper strips (Whatman filter paper) that had been previously embedded with a 0.1 M lead acetate solution and allowed to dry at 65 °C for 10 min and deposited over microplate wells. Hydrogen sulfide formation was

qualitatively measured based on the degree of blackening of the lead acetate strip and quantitatively estimated by densitometric measure of the intensity (Software "My Image Analysis v1.1" Thermo Scientific).

Killer activity was measured by the method described by Santos et al. (2009). Yeast to be tested for killer activity were inoculated in ~1-cm diameter concentrated zones onto YMA-MB plates (1 % glucose, 0.3 % yeast extract, 0.3 % malt extract and 0.5 % proteose peptone no. 3, supplemented with 30 mg/L of methylene blue, 3 % NaCl and 2 % agar) previously seeded with a lawn (5.0×10^5 cells/ml) of the sensitive yeast (*S. cerevisiae* Hansen BY4741). The sensitive strain was grown on YMA medium (YMA-MB without NaCl and methylene blue) and suspended in sterile water just before inoculation. The plates were incubated for a week at 20 °C. Killer yeasts were identified by a clear zone of inhibition surrounding them (Llorente et al. 1997).

Biomass production

S. cerevisiae CT007 was obtained as active dry yeast and rehydrated following the manufacturer's instructions. *T. delbrueckii* cultures were obtained by using an enriched must medium (12.5 % concentrated must (final concentration, 50 g/L glucose+fructose), 1 % yeast extract, 0.5 % proteose peptone no.3, pH 3.5) at 25 °C. Upon reaching the necessary cell concentration, *T. delbrueckii* was concentrated by decantation and then used as inoculum for vinifications.

Microvinifications and growth kinetics

All fermentations were prepared using the must from *Vitis vinifera* L.cv. Tempranillo grapes from El Socorro (Experimental Vineyard, Madrid, Spain) and processed accordingly to the methods described previously with slight modifications (Benito et al. 2012; Sampaio et al. 2007). Fresh must was bleeding from crushed-grapes (3.5 L) and placed in 4.9-L glass fermentation vessels, leaving enough space for carbon dioxide emission. Sulphur dioxide (40 mg/L) (Panreac, Barcelona, Spain) was added to each vessel. The sugar content was 247 g/L, yeast assimilable nitrogen 188 mg/L, pH 3.42.

By triplicate, four assays were performed: (1) inoculation with *T. delbrueckii* (Td), (2) sequential inoculation (SQ) with *T. delbrueckii* followed by *S. cerevisiae* CT007 after 15 g/L sugar consume was detected, (3) simultaneous co-inoculation (SM) with *T. delbrueckii* and *S. cerevisiae* CT007 and (4) inoculation with *S. cerevisiae* CT007 (Sc).

Cultures were adjusted in order to reach an initial cellular concentration in must of about 10^6 cells/ml for every strain, developing mixed cultures with an inocula ratio of 1:1. During co-fermentations, aliquots were taken periodically, and further tenfold dilutions were made serially. Growth kinetics were followed by plating 50 μL of the appropriate dilution on Sabouraud glucose agar with chloramphenicol (total yeast counts) and lysine media (non-*Saccharomyces* counts). Colonies were counted after growth at 30 °C for 48–72 h.

All fermentation processes were carried out at 20 °C. Once fermentation of sugars was completed (deemed to be represented by a remaining glucose+fructose concentration lower than 3 g/L), 50 mg/L of sulphur dioxide was added in potassium metabisulfite form to the wines, and they were racked and stabilised during 7 days at 4 °C, and the final product was bottled. Bottles were placed horizontally in a climate chamber TR2V120 (La Sommelie`re, Saint-Saturnin, France) at 18 °C and 70 % relative humidity. These conditions were maintained until the sensory evaluation took place.

Semi-industrial fermentations

All semi-industrial fermentations were undertaken using *V. vinifera* L. cv. Tempranillo must. Eighty kilograms of fresh crushed grapes were placed in 100 L stainless steel fermentation tanks, leaving enough space for the emission of carbon dioxide. Sulphur dioxide (40 mg/kg) was added to each. The sugar content was 247 g/L, yeast assimilable nitrogen 188 mg/L, pH 3.42.

Four assays were performed as described above for microvinifications. All fermentation processes were carried out at winery temperature of 20 °C. Once fermentation of sugars was complete (deemed to be represented by a remaining glucose fructose concentration lower than 3 g/L), the wines fermented with *T. delbrueckii* were racked and stabilized during 15 days at 4 °C, and the final product was bottled. Fifty milligrams per liter of sulphur dioxide were added in potassium metabisulfite form. Corked bottles were placed as described above. These conditions were maintained for 7 weeks until the sensory evaluation took place.

Analytical determinations of non-volatile compounds

Glucose fructose, malic acid, lactic acid, acetic acid, glycerol, pyruvic acid and colour intensity were all determined using the Y15 Enzymatic Autoanalyzer (Biosystems S.A, Barcelona, Spain). These analyses were performed using the appropriate kits supplied by the manufacturer (www.biosystems.pt).

Total acidity, pH, ethanol and density were determined following the methods in the Compendium of International Methods of Analysis of Musts and Wines (OIV 2014).

Analytical determinations of volatile compounds

Volatile compounds from microvinifications The concentration of volatile compounds (Tables 2 and S2), all of which influence wine quality, were measured at the end of alcoholic

fermentations by gas chromatography using an Agilent Technologies 6850 gas chromatograph with a flame ionisation detector (Hewlett Packard, Palo Alto, CA, USA) (Ortega et al. 2001). The apparatus was calibrated with a 4-methyl-2-pentanol internal standard. Gas chromatography quality compounds (Fluka, Sigma–Aldrich Corp., Buchs SG, Switzerland) were used to provide standard patterns. Higher alcohols were separated as described in the Compendium of International Methods of Analysis of Musts and Wines (OIV 2014). The detection limit was 0.1 mg/L. Minor compounds were quantified by gas chromatography–mass spectrometry as described by Lopez et al. (2002) with the modifications introduced by Loscos et al. (2007).

Analysis of mannoprotein content of wines Total soluble wine polysaccharides were evaluated in duplicate by using a HPLC apparatus (Surveyor Plus chromatograph, Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector (Surveyor RI Plus Detector) as reported (Quirós et al. 2012). The column employed was a 300 × 7.7 mm PL Hi-Plex Pb 8 lm (Varian, Inc., Shropshire, UK). MilliQ water was used as the mobile phase at a flux of 0.6 mL/min and a column temperature of 70 °C. The retention time valued was between 0 to 30 min.

Sensorial analysis The final wines were assessed (blind) by a panel of ten experienced wine tasters, all members of the staff of the Food Technology Department of the Polytechnic University of Madrid. Assessments took place in standard sensory analysis chambers with separate booths. Following the generation of a consistent terminology by consensus, two visual descriptors, five aromas and four taste attributes were chosen to describe the wines. Formal assessment consisted of two sessions held on different days where wine tasters tasted all fermented triplicates. The panelists used a 10 cm unstructured scale, from 0 (no character) to 10 (very strong character), to rate the intensity of ten attributes.

Statistical analysis All statistical analyses were performed using PC Statgraphics v.5 software (Graphics Software Systems, Rockville, MD, USA). The significance was set to $p<0.05$ for the ANOVA matrix F value. The multiple-range test was used to compare the means.

Results

Fermentation kinetics

Population dynamics

S. cerevisiae population showed the typical growth kinetic where, in all cases, it maintained high cell viability until the end of fermentations, both as only inoculum or as coinoculated with *T. delbrueckii* (Fig. 1). Figure 1a shows microbial kinetics of a fermentation carried out with *T. delbrueckii* by itself (Td), so total viable cells counted in Sabouraud medium are relating to the wild yeasts in the must. The sequential inoculation (SQ), in which *S. cerevisiae* was inoculated at day 4, showed a similar fermentation kinetic compared with Td fermentation, but with greater homogeneity in yeast populations between replicates (Fig. 1b). In these fermentations, non-*Saccharomyces* can be isolated until advanced stages of the process (day 17) contrary to what could be observed in the simultaneous inoculation of *T. delbrueckii* and *S. cerevisiae* (SM), where non-*Saccharomyces* can be only observed until the day 7 (Fig. 1c). Figure 1d shows the total cell count corresponding to the fermentation inoculated only with *S. cerevisiae* (Sc).

Sugar consumption and ethanol production

Figure 2 shows the different fermentation kinetics of microvinifications and semi-industrial fermentations by sugar consumption. In the case of the laboratory-scale assays, fermentations which were started with *T. delbrueckii* by itself (Td) and sequentially (SQ) with *S. cerevisiae* required 24 and 21 days, respectively, to complete fermentation, despite fermentations with *S. cerevisiae* by itself (Sc), and its simultaneous (SM) inoculation with *T. delbrueckii* only required 14 days to finish (Fig. 2a). Regarding semi-industrial fermentations, all of them required only 12 days to complete fermentation, despite fermentations started only with *T. delbrueckii* (Td and SQ) followed slower kinetics at the beginning compared with fermentations started with *S. cerevisiae* (Sc and SM) (Fig. 2b). The final alcohol content of the wines obtained in fermentations involving *T. delbrueckii* NSA-1 was lower than those only fermented by *S. cerevisiae* CT007 (Table 1). The semi-industrial fermentations confirmed this reduction, so final alcohol degree produced in different fermentations was gradually lower, depending on the higher *T. delbrueckii* presence (Supplementary material, Table S1).

Acetic acid and malic acid production

Slight differences in acetic acid production were observed between assays (Fig. 3). Figure 3a shows the acetic acid release kinetics in microvinifications, where SQ and SM fermentations produced final acetic acid concentrations ranging from 0.29 to 0.32 g/L, similar to Sc fermentations (0.31 g/L). Similar data were obtained from semi-industrial fermentations in which SQ fermentation shows the minimum acetic acid release (0.29 g/L) (Fig. 3b). SM and Sc fermentations show again similar acetic acid content (0.35 and 0.33 g/L, respectively) (Fig. 3b).

In addition, lower levels in total acidity and higher pH values in *Torulaspora* related fermentations were detected

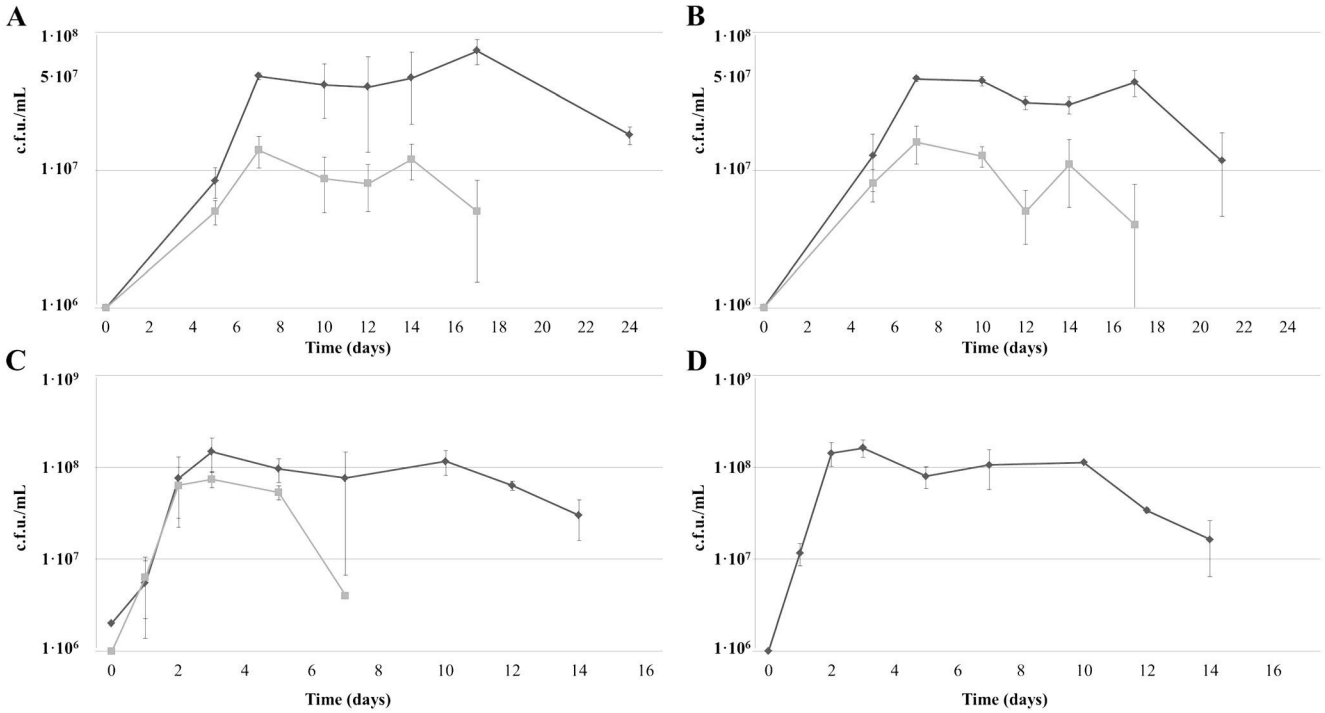

Fig. 1 Total yeast cell count (*black triangle*) and *T. delbrueckii* NSA-1 cell count (*black circle*) during fermentation. **a** Fermentation inoculated only with *T. delbrueckii* NSA-1. **b** Sequential inoculation of *T. delbrueckii* NSA-1 and *S. cerevisiae* CT007. **c** Simultaneous inoculation of *T. delbrueckii* NSA-1 and *S. cerevisiae* CT007. **d** Fermentation inoculated only with *S. cerevisiae* CT007

(Table 1). It also could be related to the higher malic acid consumption by *T. delbrueckii*.

Fermentations involving *T. delbrueckii* NSA-1 consumed part of the malic acid present in the must, which showed an initial malic acid content of 1.77 g/L. The final values in fermentations involving *T. delbrueckii* NSA-1 were lower than Sc fermentation ones (Table 1), detecting a maximum malic acid reduction rate of 13.56 % in Td fermentation and a 4.52 % of malic acid reduction in Sc fermentation. Table 1

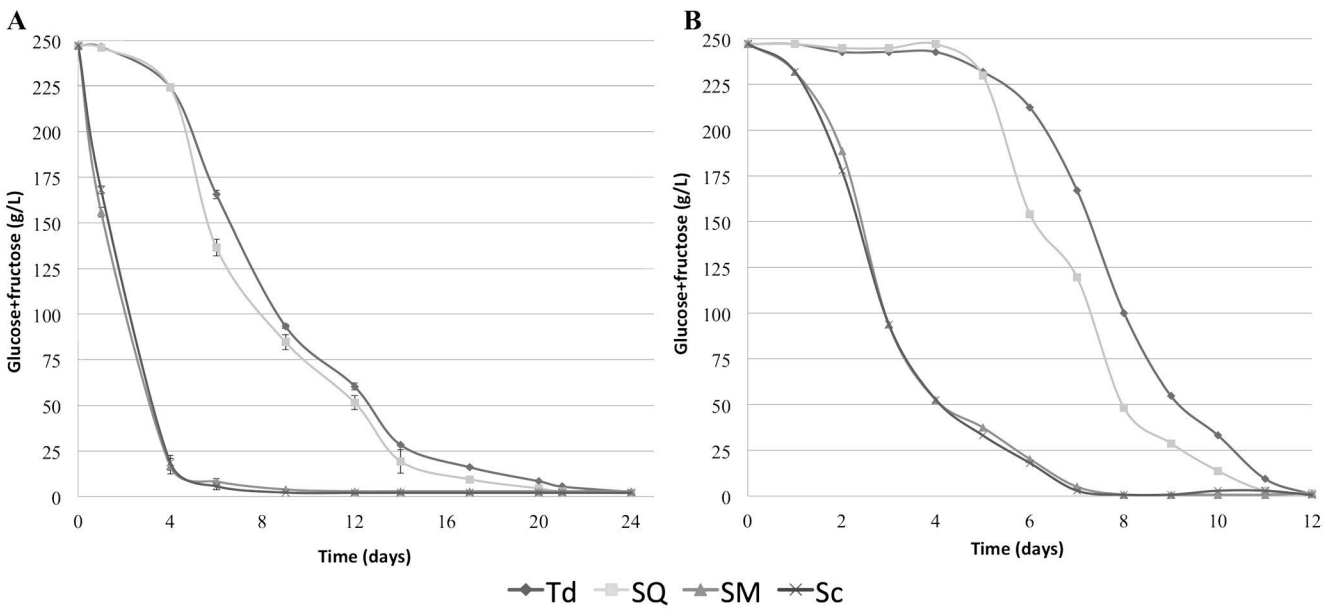

Fig. 2 Change in glucose fructose concentration of the studied Tempranillo-based wines during fermentation with *T. delbrueckii* NSA-1 alone (*Td*); sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (*SQ*); simultaneous fermentation with *T. delbrueckii* NSA-1 *S. cerevisiae* (*SM*); fermentation with *S. cerevisiae* CT007 alone (*Sc*). **a** Laboratory-scale assays; **b** semi-industrial-scale assays

Table 1 Analytical results for the wines produced by the different fermentation systems

Assays	Glucose fructose (g/l)	Acetic acid (g/l)	Malic acid (g/l)	Lactic acid (g/l)	Glycerol (g/l)	Free SO2 (mg/L)	Total SO2 (mg/l)	Total acidity(g/l)	Alcohol (%*v/v*)	pH	Colour intensity
Td	2.49±0.47a	0.37±0.02a	1.53±0.03a	0.11±0.02a	6.70±0.03a	25.13±3.21a	82.13±3.21a	6.62±0.08a	14.38±0.07a	3.56±0.01a	3.16±0.05a
SQ	1.99±0.37a	0.29±0.02b	1.57±0.04b	0.10±0.02a	6.71±0.02a	23.42±1.21a	79.31±2.33ab	6.67±0.06a	14.39±0.03a	3.54±0.02c	3.09±0.03a
SM	.88±0.13b	0.32±0.02b	1.61±0.02b	0.09±0.01a	6.63±0.02b	26.38±2.46a	77.13±2.21ab	6.75±0.02b	14.46±0.02b	.53±0.02c	2.96±0.02b
sss	2.03±0.22a	0.31±0.01b	1.69±0.02c	0.09±0.01a	6.63±0.01b	24.13±1.76a	4.13±2.02b	6.77±0.01b	14.53±0.02b	.51±0.04c	2.77±0.02c

Results represent the mean SD for three replicates. Means in the same row with the same letter are not significantly different ($s<0.05$)

Td T. delbrueckii NSA-1 alone, *SQ* sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007, *SM* simultaneous fermentation with *T. delbrueckii* NSA-1+*S. cerevisiae* CT007, *Sc* fermentation with *S. cerevisiae* CT007 alone

shows final concentrations of lactic acid; the absence of malolactic fermentation confirmed that no contamination by lactic acid bacteria occurred.

Pyruvic acid and glycerol production

S. cerevisiae CT007 by itself (Sc) and SM fermentation showed maximum pyruvic acid production at fourth day, reaching 111 and 141 mg/L, respectively, in microvinifications (Fig. 4a). Td and SQ fermentations showed higher values with maximum figures of 156 and 143 mg/L, respectively, at day 6. Similar values and kinetics can be observed in the semi-industrial fermentations where *T. delbrueckii* contributed to the pyruvic acid production obtaining its maximum values times depending on the different fermentation kinetics (Fig. 4b).

The glycerol content in Td and SQ fermentations was also slightly higher than the one observed in Sc and SM fermentations in microvinifications (Table 1) and semi-industrial trials (Supplementary material, Table S1).

Volatile compounds

Table 2 shows that fermentations involving *T. delbrueckii* produced lower concentrations of higher alcohols; nevertheless, all fermentations produced these compounds in moderate quantities. A similar effect was observed in the case of esters and fatty acids. Some compounds such acetaldehyde, diacetyl and ethyl acetate were detected in higher values when *Torulaspora* was used alone. These results were confirmed in semi-industrial-scale vinifications (Supplementary material, Table S2). No differences of terpenic compounds between assays (Supplementary material, Table S2) were found in this trial.

Sensorial analysis

Figure 5 shows a "spider web" diagram for the average scores of some olfactory and taste attributes. Light differences in the perception of acidity were recorded. Colour intensity perception was higher in those fermentations in which *T. delbrueckii* NSA-1 took place. Fermentation with *S. cerevisiae* CT007 alone produced slightly stronger sensations of oxidation. None of the wines that involved fermentation with *T. delbrueckii* had any perceptible organoleptic problems; indeed, sequential and mixed fermentations received the best scores from all tasters. The greatest virtue attributed to SQ fermentation was the complexity and structure of its mouthfeel properties.

Mannoproteins content in semi-industrial fermentations

Final content of mannoproteins in semi-industrial scale fermentation in tanks containing 80 kg of crushed grapes were

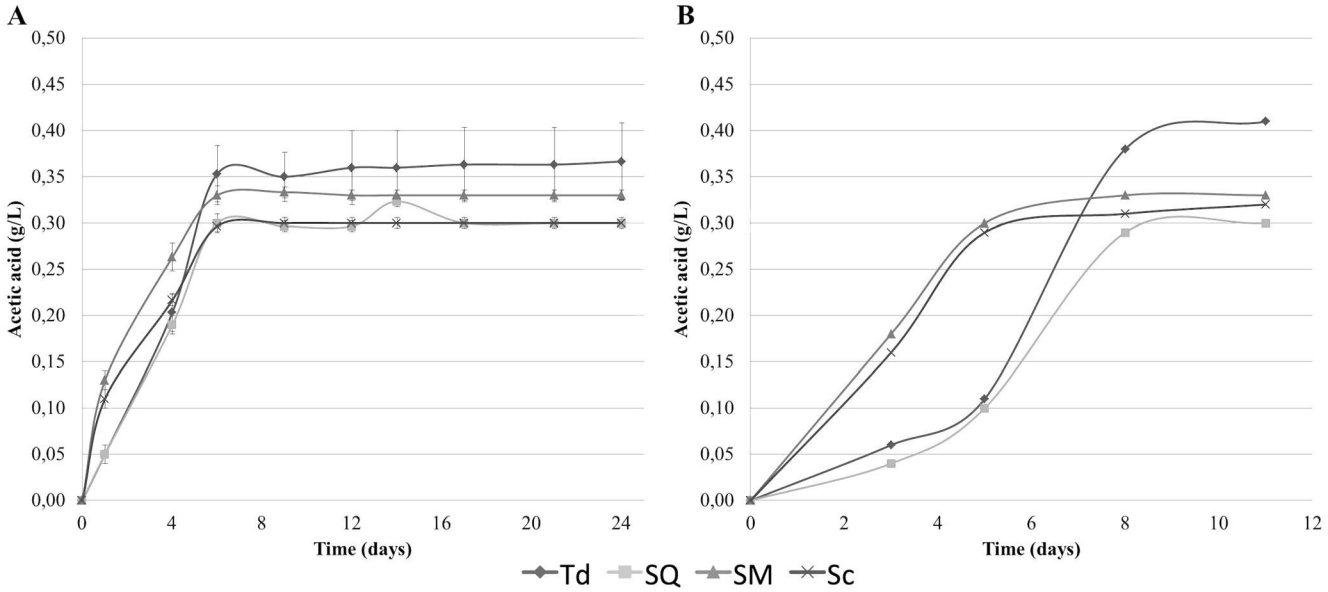

Fig. 3 Change in acetic acid concentration of the studied Tempranillo-based wines during fermentation with *T. delbrueckii* NSA-1 alone (*Td*); sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (*SQ*); simultaneous fermentation with *T. delbrueckii* NSA-1 + *S. cerevisiae* (*SM*); fermentation with *S. cerevisiae* CT007 alone (*Sc*). **a** Laboratory-scale assays; **b** semi-industrial scale assays

analysed. In Fig. 6, the increase of mannoproteins can be seen in the fermentations in which *T. delbrueckii* acts for longer, especially in controlled sequential fermentation.

Discussion

Similar results in fermentation kinetics and population dynamics (Fig. 1) can be seen in recent analogous studies using non-*Saccharomyces* yeasts, where simultaneous inoculation of both, *S. cerevisiae* and non-*Saccharomyces* strains, limited the presence of non-*Saccharomyces* to the early stages of fermentation (Azzolini et al. 2012; Oro et al. 2014). In this work, the studied yeast strains were observed to present killer phenotype and were active against the sensitive strain used as control (*S. cerevisiae* BY4741). However, there was no cross-activity between them, so killer activity was not considered as a relevant feature in the growth kinetics of both strains.

The sugar consumption results showed in this work (Fig. 2) agree with the lower fermentative power of *Torulaspora* spp.

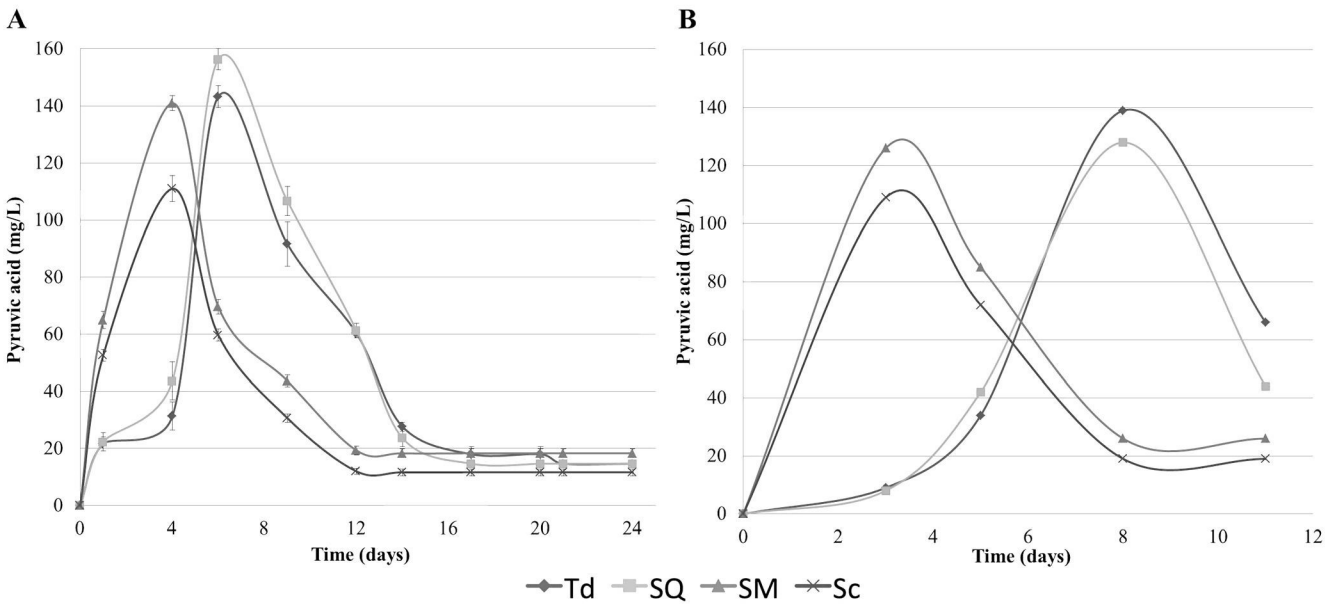

Fig. 4 Change in pyruvic acid concentration of the studied Tempranillo-based wines during fermentation with *T. delbrueckii* NSA-1 alone (*Td*); sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (*SQ*); simultaneous fermentation with *T. delbrueckii* NSA-1 + *S. cerevisiae* (*SM*); fermentation with *S. cerevisiae* CT007 alone (*Sc*). **a** Laboratory-scale assays; **b** semi-industrial scale assays

Table 2 Volatile compounds (micrograms per liter) detected in the different fermentations

Compounds	Sc	SM	SQ	Td
1-Hexanol	1,835.33±80.03b	1,983.00±45.90a	1,557.00±72.63c	1,859.00±77.32ab
3-Hexanol	684.67±7.02a	680.67±21.94a	457.67±17.95c	564.33±23.59b
Isoamylalcohol	391,316.33±13,882.09a	403,590.33±4,815.73a	334,420.00±9,634.85b	386,564.67±9,624.89a
Isobutanol	55,502.00±1,213.69b	60,904.33±1,608.61a	54,783.00±1,371.60b	61,803.33±1,533.15a
Alcohols	449,337.99±12,961.9b	467,158.33±4,523.64a	391,217.67±8,922.84c	450,791.33±8,930.99b
Acetaldehyde	332.27±631.43a	7,332.27±631.43a	7,254.34±672.34a	11,342.13±792.23b
Diacetyl	1,243.45±65.13a	1,425.32±88.43b	1,223±77.34a	1,634.43±104.47c
Carbonyl compounds	8,575±584.41b	9,846±687.29b	8,477.34±622.67c	1,2976±741.61a
Ethyl acetate	21,365.23±1,365.45b	23,456.34±1,567.76ab	21,897.65±1,645.54b	5,764.26±1,876.54a
Ethyl butyrate	157.33±4.73b	182.00±5.29a	144.67±7.02c	181.67±6.51a
Ethyl decanoate	86.33±4.52a	95.00±6.00a	74.33±4.06b	80.00±8.73ab
Ethyl hexanoate	613.67±17.16a	652.33±16.56a	339.67±20.60c	462.33±23.29b
Ethyl lactate	3,700.33±121.38a	3,711.33±104.31a	2,832.67±120.02c	2,832.67±120.02c
Ethyl octanoate	372.00±33.15a	425.67±46.74a	297.00±12.53b	377.67±36.74a
Isoamyl acetate	1,381.67±43.25a	1,073.33±47.48b	974.00±12.53c	1,136.00±79.54d
Esters	2,7676.23±1,200.65bc	29,596±1,396.32b	26,559.99±1,494.77c	31,156.93±1,706.91a
hexanoic acid	3,614.33±140.47a	3,521.33±151.64a	3,154.00±157.29b	3,383.33±161.98ab
Isobutyric acid	3,614.33±140.47a	3,521.33±151.64a	3,154.00±157.29b	3,383.33±161.98ab
isovaleric acid	385.67±7.51a	368.00±4.58b	331.33±13.61c	363.67±6.66b
octanoic acid	9,919.33±74.59a	10,125.67±145.29a	6,703.00±220.96c	8,544.00±224.01b
valeric acid	598.67±12.22a	584.33±20.40a	531.33±25.20b	567.67±11.72ab
2-Phenylethanol	54,308.67±1,151.98a	53,194.00±2,022.95ab	52,531.67±1,170.21ab	51,485.33±719.18b
2-phenylethyl acetate	8.00±1.00a	7.67±2.31a	7.67±1.53a	7.00±1.00a
Phenols	8.00±1.00a	53,201.67±2,022.82ab	52,539.34±1,170.13ab	51,492.33±719.13b

Results represent the mean SD for three replicates. Means in the same row with the same letter are not significantly different ($s<0.05$)

T. delbrueckii NSA-1 alone (Td); Sequential fermentation with *T. delbrueckii* NSA-1 followed by *S. cerevisiae* CT007 (SQ); Simultaneous fermentation with *T. delbrueckii* NSA-1+*S. cerevisiae* CT007 (SM); fermentation with *S. cerevisiae* CT007 alone (Sc)

compared with *S. cerevisiae* reported by other authors (Bisson and Kunkee 1991; Jolly et al. 2006), due to the fact that, in the last stages, only *Saccharomyces* was detected (Azzolini et al. 2012). The slower kinetics of *T. delbrueckii* fermentations (Fig. 2a) was attributed to a high nutrient demand by these species that limited the later *S. cerevisiae* fermentation activity (Romano et al. 2003). Data obtained for fermentation kinetics in semi-industrial fermentations confirm this trend. The fact

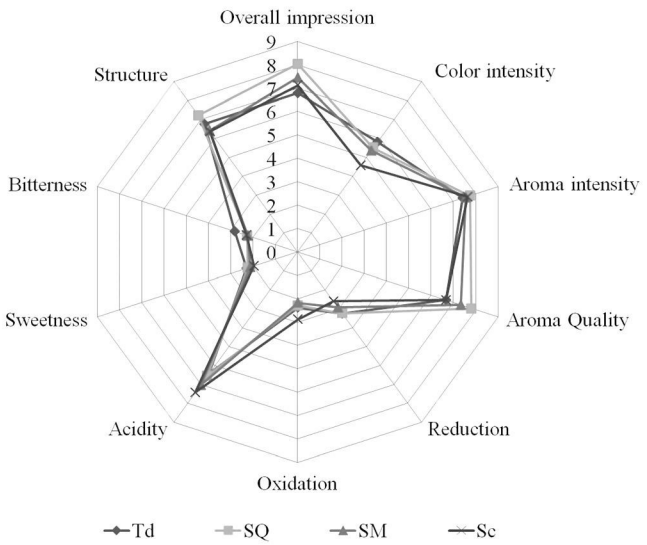

Fig. 5 Taste and olfactory attribute scores for the final wines

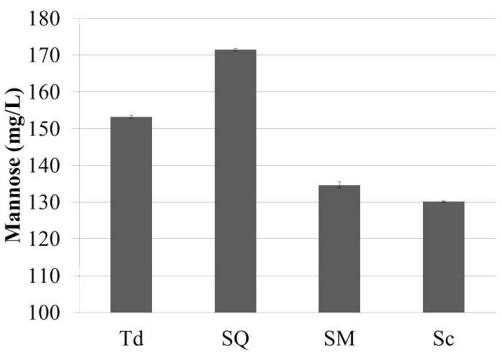

Fig. 6 Mannoprotein content (milligrams per liter of mannose) of wines fermented at semi-industrial scale with: *S. cerevisiae* CT007 (*Sc*); *T. delbrueckii* NSA-1 and *S. cerevisiae* CT007 by using simultaneous inoculation (*SM*); sequential inoculation (*SQ*); and *T. delbrueckii* NSA-1 alone (*Td*)

that the semi-industrial fermentation kinetics was faster than microvinifications (Fig. 2) can be explained because of the different composition of the must. In microvinifications, the fermentative media contained only fresh must and must with crushed grapes in semi-industrial fermentations, which contribute to nutrient enrichment of musts.

Several authors argue the usefulness of non-*Saccharomyces* yeast in the production of lower concentrations of alcohol in wines (Contreras et al. 2014; Kutyna et al. 2010), reporting reductions higher than 1 % in final alcohol content. These previous results agree with the lower final alcohol content of the wines produced in fermentations involving *T. delbrueckii* NSA-1 in this work (Table 1); however, in this assay, the ethanol reduction was lower than 0.2 %. Sugar consumption could also be used to produce alternative compounds to ethanol, such as glycerol or pyruvic acid, or to increase the yeast biomass by *T. delbrueckii* due to its reported lower Crabtree effect (Bely et al. 2008; Merico et al. 2007).

Higher acetic acid values from Td fermentations (Table 1) than the others, both in microvinifications and in semi-industrial fermentations, can be attributed to the wild *Saccharomyces* yeasts that finish the fermentation, observing the increase in the release of acetic acid at the beginning of tumultuous fermentation (Fig. 3) and confirming the usefulness of selected strains to reduce the acetic acid content in wines. One of the questions raised by winemakers is the excessive increase of acetic acid in wines with high presence of non-*Saccharomyces* yeasts (Jolly et al. 2014). Our results show that using *T. delbrueckii* in mixed fermentations does not cause an increase of acetic acid (Table 1), according to the results reported in similar studies (Azzolini et al. 2012). Other authors also described *T. delbrueckii* as a low acetic acid producer compared with most non-*Saccharomyces* yeasts (Bely et al. 2008; Moreno et al. 1991; Renault et al. 2009).

The higher decrease in malic acid content observed in the trials involving *T. delbrueckii* (Table 1) agrees with the reported by other authors who confirm that malic acid can be metabolised by several yeast species (Benito et al. 2013, 2014a, 2014b; Suárez-Lepe et al. 2012; Su et al. 2014) in levels lower than 20 %, unless *Schizosaccharomyces* species are used.

Previous pyruvic acid-based selection studies on *S. cerevisiae* strains returned maximum values of 60–132 mg/L after 4 days of fermentation (Morata 2004), values below those obtained in the present study with *T. delbrueckii* fermentations (Fig. 4a). A strong correlation has been reported between the amount of pyruvic acid released into the medium and the formation of vitisin A (Morata et al. 2003, 2012) which is also related to colour stability. Other authors have reported before a high production of other acid in yeast metabolism by *T. delbrueckii* such as succinic acid (Ciani and Maccarelli 1998). Different non-*Saccharomyces* yeasts have been found to have influence in intensity and stability of wine colour (Benito et al. 2011, 2014c; Morata et al. 2012). Final OD values in colour intensity of 3.16, 3.09, 2.96 and 2.77 were returned for Td, SM, SQ and Sc fermentations, respectively (Table 1). The formation of highly stable pigments such as vitisin A, due to the higher pyruvic acid formation, could explain these chromatic differences between wines. Also, colour material absorption could be different between species and strains (Morata et al. 2005). Furthermore, higher total sulphur dioxide levels (Table 1) in fermentations involving *T. delbrueckii* could also be explained by higher combinations of anthocyanins with pyruvic acid during fermentation (Morata et al. 2003).

The increase of glycerol content in wines is one of the most recognised contributions of non-*Saccharomyces* species to the quality of wines (Jolly et al. 2006). However, some authors reported that an increase in glycerol production is usually linked with a rise in acetic acid production (Prior et al. 2000), which can be detrimental to wine quality. This fact was observed in *Candida stellata* strains that can produce elevated concentrations of glycerol (10 to 14 g/L) compared with *S. cerevisiae* (4 to 10 g/L); on the contrary, our results confirm that these facts seem to be irrelevant in the case of *T. delbrueckii*.

The values observed in pyruvic acid and glycerol production could indicate that *T. delbrueckii* possesses a highly active glyceropyruvic pathway (Ciani and Maccarelli 1998; Renault et al. 2009). Besides, some authors have been reported that there is a big difference in glycerol production depending on strain level (Loira et al. 2012).

The detected lower production of higher alcohols by *T. delbrueckii* could have increased the varietal Tempranillo aroma perception. Different non-*Saccharomyces* yeasts produce different levels of higher alcohols (Lambrechts and Pretorius 2000; Romano et al. 1992). This can be important because a large concentration of higher alcohols can generally not be desired, whereas lower values can contribute to wine complexity (Romano and Suzzi 1993). Non-*Saccharomyces* yeasts often form lower levels of these alcohols than *S. cerevisiae*, but there is great strain variability (Romano et al. 1992; Zironi et al. 1993). The higher values detected in acetaldehyde, diacetyl and ethyl ethanol when *Torulaspora* was used alone could be attributed to wild high fermentative non-selected yeasts involved in a spontaneous process. Other authors have reported a higher production of terpenic compounds by *T. delbrueckii* in Muscat variety (King and Dickson 2000). In contrast, our results show no differences between assays in this kind of compounds (Supplementary material, Table S2). However, this ability is attributed to specific strains and *T. delbrueckii* NSA-1 does not possess the terpenic-related enzymatic properties that were analysed (β-glucosidase, β-D-xylosidase and α-L-arabinofuranosidase).

The recorded differences in acidity perception could be related to the small malic acid consumption detected in

fermentations in which *T. delbrueckii* NSA-1 was involved and to the lower total acidity levels obtained in these wines (Table 1). Differences in colour intensity perception could be partially explained because of the higher pyruvic acid content detected and its influence in high stable colour forms (Benito et al. 2011). Other authors described that wines fermented by coinoculation with *T. delbrueckii* and *S. cerevisiae* are better than the regular *S. cerevisiae* control for the varieties Sauvignon Blanc, Chenin Blanc and Amarone (Azzolini et al. 2012; Jolly et al. 2003). In this work, a similar effect was found for the Tempranillo variety. The tasters perceived higher aroma quality in the specific cases of SQ and SM fermentations, but no strong difference in aroma compounds was observed. This could be explained due to a lower higher-alcohol content which generally overlays other minor compounds that contribute to the wine aroma complexity. Mannoproteins are one of the main microbial metabolites related with the complexity of wine mouthfeel properties. The better mouthfeel structure of SQ fermentations that contributed to the higher overall score could be related to this fact (Fig. 6). In addition, a higher perception in sweetness was detected, probably due to the malic acidity consumed by *T. delbrueckii*, but also influenced by higher levels of mannoproteins in wines.

One of the main contributions of non-*Saccharomyces* yeasts during wine fermentation are their repercussion on the mouthfeel properties (Suárez-Lepe and Morata 2012). Macromolecules derived from the yeast cell wall, particularly mannoproteins, have capital importance in the mouthfeel properties (Gonzalez-Ramos et al. 2008), and enological empirical experience carried out to date with *T. delbrueckii* talks about a remarkable complexity and roundness in mouthfeel (Guadalupe et al. 2007). Recently, *T. delbrueckii* has been described as a wine yeast with a higher content of wall polysaccharides (Domizio et al. 2014).

This study contributes to confirm the role of non-*Saccharomyces* in wine fermentation by analysing metabolic and physiological properties of a new industrial strain of *T. delbrueckii*. A significant effect in some major aroma compounds (higher alcohols and esters), as in pyruvic, malic and acetic acids and in alcohol content were found in microvinifications carried out with *T. delbrueckii* NSA-1 industrial strain using different combined fermentation strategies, concluding that sequential fermentation is the most appropriate. Scaling assays for validating the industrial use of yeasts are a key factor and the bottleneck of the yeast selection process. In this study, we validate the use of the new *T. delbrueckii* NSA-1 strain in a semi-industrial assay, and similar results can be found for all parameters analyzed. Furthermore, sensorial analysis of these semi-industrial fermentations emphasized the improvement of mouthfeel properties in fermentations in which *T. delbrueckii* was involved. This fact could be explained, aside from the chemical and aromatic properties already mentioned, because of the increase in mannoprotein content of these wines. The use of non-*Saccharomyces* yeast in winemaking implies its adaptation to a cellar environment, so semi-industrial and industrial assays should be considered as important and ought to be included in scientific reports.

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